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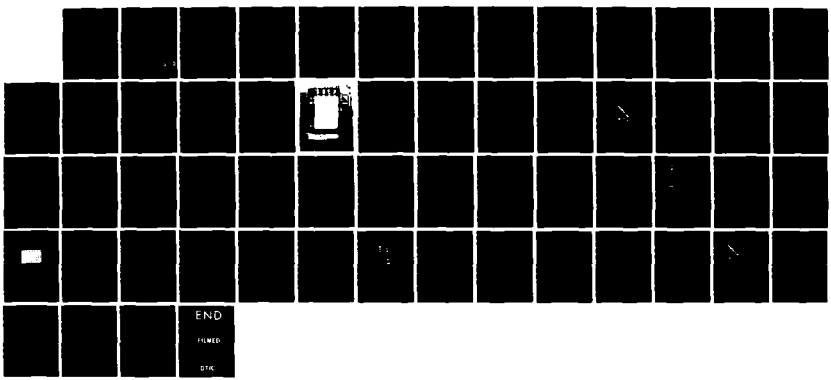

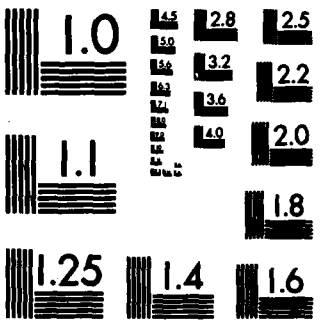

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Mechanisms of Bunyavirus Virulence: A Genetic Approach

Annual Report

Neal Nathanson, M.D.

December 1983
(02 Year)

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20. (Abstract)

The construction and characterization of hybridomas making monoclonal antibodies against La Crosse and Tahyna viruses is described. These antibodies have been characterized by the following tests: (2) ELISA; (3) neutralization; (5) hemagglutination inhibition (HI); (4) cross reactivity against California serogroup viruses; (5) immunoprecipitation of viral proteins. These antibodies have been shown to be useful tools for the classification of new viral isolates and for the phenotyping of reassortant viruses. They have been used to select variant viruses which can escape neutralization.

The virulence of California serogroup viruses is being studied by genetic analysis. (a) Two parent viruses have been selected to represent a virulent prototype (La Crosse original) and an avirulent prototype (Tahyna 181/57). (b) The pathogenesis of these two viruses has been studied by infections of suckling mice, and it has been found that the neuroinvasive virulent virus replicates well in striated muscle with subsequent viremia, while the avirulent virus does not. (c) Reassortants have been constructed from these two parents and partially phenotyped. (d) Variant viruses, selected with neutralizing monoclones are under study for reduced virulence.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW) Publication No. (NIH) 78-23, Revised 1978.

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1. Introductory note: Scope and Duration

As negotiated, the budget for this contract for the 02 year, 12-01-82 through 11-30-83, is summarized below.

Components of Budget	02 Year
Total Direct Costs	65,807
Indirect Costs (65% TDC)	32,537
Total Contract	98,344

Since it was clear that only a part of the original proposal could be funded on a contract of this size, we have selected the DEVELOPMENT, CHARACTERIZATION, AND UTILIZATION OF HYBRIDOMAS as the focus for this project. Nevertheless, the original 3 investigators are collaborating on this aspect of the program.

Neal Nathanson	Professor	Pathogenesis and immunology
Francisco-Gonzalez	Assistant Professor	Hybridomas and variant viruses
Jon Gentsch	Assistant Professor	Genetics and protein chemistry
Robert Janssen	Post doctoral Fellow	Pathogenesis

Also, it should be noted that this report was written in January, 1983, when the contract had run only 1 month of the 02 year. Work reported was mainly done in the 01 year.

2. Hybridomas against LaCrosse and Tahyna viruses

(a) Papers

Much of our work to date is summarized in two papers. These papers are duplicated at the end of this Progress Report and details will not be repeated in the following summary.

(b) Uses of hybridomas

The central theme of our studies is to use the California virus system as a model to study the factors which determine the outcome of acute viral encephalitis. Among these factors are virus determinants (neuroinvasiveness and neurovirulence) and host determinants (recovery from infection and protection against subsequent infection). We plan to use monoclonal antibodies as a tool to investigate several aspects of this model.

(i) To make reassortant viruses from virulent from virulent and avirulent parent viruses, monoclones can be used to rapidly phenotype putative reassortants from both G1 and N proteins.

(ii) To make antigenic variants of parental virus growth in the presence of monoclones will select efficiently for variant viruses. These variant viruses can be used for two distinct purposes: (a) To determine if variants show changes in their virulence, which could occur if the G1 protein is an important determinant of virulence. (b) To group the monoclones themselves according to their reactivity with a panel of variant viruses.

(iii) To identify and map biological functions of the viral glycoproteins. Important functions associated with glycoproteins of enveloped viruses are: binding to erythrocytes (hemagglutination), binding to host cells (neutralization), and fusion of membranes (hemolysis, cell fusion, infectivity).

(iv) To determine the protective role of antibodies directed against antigenic determinants of California encephalitis virus glycoproteins.

In other enveloped viruses antibodies against certain sites on the glycoprotein will neutralize, but the efficiency may differ markedly, depending on whether the site is involved in attachment or in fusion. Also, there may be a synergistic effect of neutralizing antibodies against two different antigenic sites.

Antibodies which fail to neutralize may have several different effects: in some cases non-neutralizing antibodies block neutralization, while in other instances they are protective in vivo.

Finally, the properties of the antibody itself (avidity, complement fixation, ability to mediate virolysis and cytolysis, and the like) may influence its protective efficiency. Such questions can be studied with monoclonal antibodies much more precisely than could ever be accomplished with polyclonal antisera.

(c) Immunization of mice and construction of hybridomas

LaCrosse and Tahyna viruses were selected for this study because they represent antigenically distinct strains with relative differences in virulence in mice, LaCrosse being the more neuroinvasive (kills after ip injection) and Tahyna the more avirulent (fails to kill after ip injection above 2 weeks of age). Also, it had been shown that reassortants could readily be made between these two viruses.

To immunize mice, advantage was taken of the fact that LaCrosse and Tahyna viruses produce active infections in mice; intraperitoneal or intracerebral injection was used, to initiate a severe infection with some deaths, and survivors were used. A booster injection of virus was given and 2-4 days later mice were sacrificed and spleen cells prepared.

Spleen cells from LAC or TAH immunized mice were fused with a BALB/c myeloma line (P3 x 63 clone 653 which is a nonsecretor. In the purine salvage pathway; this function is provided by the lymphocyte partner in the hybrid cell. A mixture of spleen:myeloma cells at a 10:1 ratio was made and PEG 1000 used as fusing agent. The mixture was plated in micro wells, 5×10^5 cells per well.

After 2-3 weeks of incubation, wells with visible colonies were tested for anti-viral antibody in ELISA assay, using partially purified virus as antigen. Positive cultures were cloned in 0.25% agarose and individual colonies were transferred to flasks and again tested for antibody. Hybridoma cells were maintained in 15% serum and supernates collected as a cell culture source of monoclonal antibody. Hybridoma cells were maintained in 15% serum and supernates collected as a cell culture source of monoclonal antibody. For high titer preparations, 10^7 hybridoma cells were injected ip into Pristane-primed BALB/c mice and ascitic fluids collected 1-2 weeks later. Antibody titers of ascitic fluids were usually about 100-fold higher than titers of tissue cultures supernates. For neutralization, HI, CF, and ELISA, the ascitic fluid served well, but tissue culture supernate or purified immunoglobulin was required for clean immunoprecipitations. Cells stored well in a serum-DMSO mixture in liquid N2.

(d) Characterization of hybridomas

To characterize the LAC and TAH hybridomas, each was tested in ELISA, neutralization (N), and hemagglutination (HI) systems, against each of 11 California serogroup viruses.

In addition, each monoclone was used to immunoprecipitate virus proteins from an S35 animo acid labelled lysate of infected cells, and each was typed as to immunoglobulin class by an RIA.

The essential results are set forth in the TABLE 1 and may be summarized (See Appendix 1 and 2 for further detail):

(i) Of 23 monoclones, 15 were directed against the G1 glycoprotein and 8 against the N nucleoprotein, while none were against the G1 glycoprotein or the L polypeptide.

(ii) Of the 15 G1 clones, 11 both neutralized and had HI activity, one had HI activity only, and 3 were neutralization and HI negative. From this it was inferred that the G1 glycoprotein had at least two antigenic sites. One site is postulated to bind to receptors on both erythrocytes and substrate cells, accounting for the concordance of neutralization and HI results. The other site appears uninvolved in attachment to receptors.

(iii) Of the 15 G1 clones 4 were type-specific (group A/1) i.e., reacted with the immunizing virus only, 3 were almost type-specific (group A/2 or C), while 8 were cross-reactive (group B or D).

(iv) None of the 8 nucleocapsid clones showed neutralizing or HI activity, as expected.

(v) Of the 8 nucleocapsid clones, only one was type-specific, i.e., was a group C clone, while 7 were cross-reactive (group C/D or D).

(vi) The 15 G1 clones were isotyped as IgG1 (7 clones), IgG2a (6 clones), or IgG2b (2 clones). By contrast, the 8 nucleocapsid clones were IgM (5 clones), Ig2a (2 clones), or undetermined (1 clone).

(e) Additional anti-G1 hybridomas

To insure a more complete delineation of the antigenic sites on the important G1 glycoprotein, additional hybridomas have been made to enlarge our basic panel. These are summarized in TABLE 2.

(f) Attempts to construct anti-G2 hybridomas

The functions of the smaller bunyavirus glycoprotein (G2) are presently unknown, nor is it clear whether anti-G2 antibodies may play a role in in vivo protection or neutralization. Efforts to date to construct hybridomas against G2 have not been successful, but will be summarized briefly, since we believe this is an important problem which deserves continued effort.

(i) Hyperimmunization. Mice were immunized by a more extensive schedule than previously used, with an initial ip immunization with schedule than previously used, with an initial ip immunization with LAC virus (BHK cell lysate), followed by two boosters with gradient purified virus, given ip or sc with or without

complete Freund's adjuvant. Two to 4 days after the last boost, spleens were obtained for fusion (see Appendix I). This protocol yielded hybridomas selected by ELISA screen, but on immunoprecipitation clones were either anti-G1 or anti-nucleocapsid.

TABLE 1. CHARACTERISTICS OF 23 MONOCLONAL ANTIBODIES AGAINST LaCrosse (LAC) and Tahyna (TAH) VIRUSES

Clone No.	Immunizing Virus	Protein Precipitated	Ig Class	Group	Type-Specific Cross-Reactive	Serological Test		
						ELISA	NT	HI
807-09	LAC	G1	IgG2a	A	S	+	+	+
807-15	LAC	G1	IgG2b	A	S	+	+	+
807-18	LAC	G1	IgG1	A	S	+	+	+
807-35	LAC	G1	IgG1	A	S	+	+	+
807-31	LAC	G1	IgG1	A	S	+	+	+
807-12	LAC	G1	IgG2a	B	C	+	+	+
807-22	LAC	G1	IgG2a	B	C	+	+	+
807-33	LAC	G1	IgG2a	B	C	+	+	+
807-25	LAC	G1	IgG2b	C	S	+		
807-26	LAC	G1	IgG2a	C	S	+		
807-21	LAC	G1	IgG2a	C	C	+		
807-13	TAH	G1	IgG1	B	C	+	+	+
813-48	TAH	G1	IgG1	B	C	+	+	+
813-77	TAH	G1	IgG1	B	C	+	+	+
814-443	TAH	G1	IgG1	D	C	+		+
820-374	LAC	Nc	IgM	E	C	+		
807-28	LAC	Nc	IgG2a	E	C	+		
807-32	LAC	Nc	IgM	E/F	C	+		
807-13	LAC	Nc	IgM	E/F	C	+		
807-02	TAH	Nc	IgG2a	E	S	+		
814-08	TAH	Nc	?	E	C	+		
814-48	TAH	Nc	IgM	F	C	+		
814-87	TAH	Nc	IgM	F	C	+		

Nc, nucleocapsid. NT, neutralization test.

TABLE 2. ADDITIONAL MONOCLONAL ANTIBODIES AGAINST LAC VIRUS

data of F. Gonzalez and J. Gentsch

Clone No.	Immunizing Virus	Protein Precipitated	Serological Tests		
			ELISA	NT	HI
807-05	LAC	G1	+	-	*
807-07	LAC	G1	+	-	*
807-17	LAC	G1	+	-	*
813-57	TAH	G1	+	-	*
813-71	TAH	G1	+	-	*
813-72	TAH	G1	+	-	*
820-260	LAC	G1	+	+	*
900-03	LAC	G1	+	+	*
900-04	LAC	G1	+	-	*
900-05	LAC	G1	+	-	*
900-08	LAC	G1	+	-	*
900-11	LAC	G1	+	-	*
900-13	LAC	G1	+	-	*
900-19	LAC	G1	+	-	*

* HI: not yet run. Isotyped cross-reactivity to be determined. NT, neutralization test.

(ii) Enriched G2 fraction. LAC virus was gradient purified and disrupted with 0.5% triton x100. The disrupted virus was adsorbed extensively with anti-G1 anti-Nc immunoglobulin-sepharose made by coupling the purified IgG fraction of monoclonal antibody supernatants to cyanogen bromide-activated sepharose 4B. The supernate from this procedure was dialysed extensively to remove Triton and then tested as an ELISA antigen. It was positive with a hyperimmine polyclonal anti-LAC virus mouse serum but negative with high concentrations of monoclonal antibodies against G1 or Nc protein in this antigen preparation.

Mice, immunized as described above, were used for a fusion. Individual hybrid clones were then screened using two ELISA antigens in separate test; first, gradient-purified whole LAC virions, and second, the putative enriched G2 fraction. Clones were found which gave positive (albeit weak) reactions on the "G2" antigen and which were only weakly positive on the whole virus antigen. Six of these clones were grown in cell culture and the antibodies purified from cell culture supernatants or ascites fluids were tested by immuno-precipitation of S35-labelled LAC virus proteins.

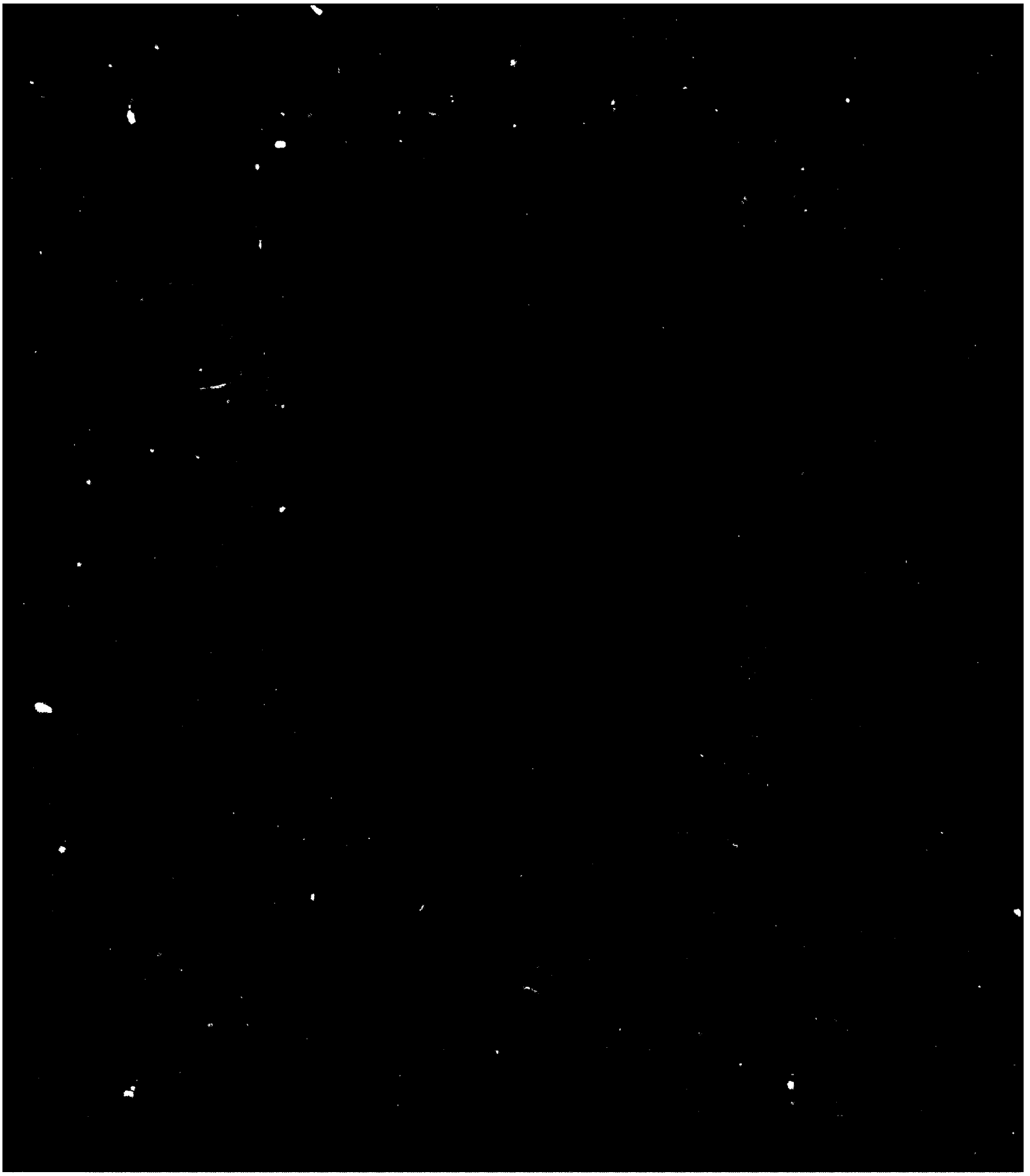
Unfortunately, none of these precipitated any LAC protein, even though they continued to register weakly positive against whole LAC virus ELISA antigen. The reason for these "false positive" results is not clear, but it appears that other approaches are required. However, the enriched "G2" antigen is still available for screening in these future attempts. It is also unclear why it has been so difficult to isolate hybridomas secreting anti-G2 antibodies; to date, two other laboratories which have made LAC virus hybridomas have failed to isolate such hybridomas (Kingsford, personal communication; Grady, personal communication).

(g) Phenotyping of reassortant viruses with monoclones and by PAGE

A major use for the monoclonal antibodies was to phenotype reassortant viruses. Using authenticated reassortants (courtesy of D. Bishop), prototype tests were conducted by ELISA, neutralization, HI, and CF methods. These different tests produced congruent results and a subset of the data is shown in the references. Clearly, selected monoclones are capable of distinguishing LAC and TAH G1 and LAC and TAH nucleocapsid proteins, and therefore can be used to phenotype reassortants for the products of the M RNA and S RNA genes.

An alternative method for phenotyping the G1, G2, and Nc proteins is based on the observation by Gentsch and Bishop that these proteins migrated slightly differently when certain California serogroup viruses were run in adjacent lanes in standard PAGE system, such as that we have been using (see reprint attached). Specifically, when authenticated LAC-TAH reassortants (courtesy of

D.H.L. Bishop) were run in adjacent lanes, there were clearcut differences between LAC and TAH viruses for G1, G2, and Nc proteins (Figure 1).



Thus, we now have two independent methods for phenotyping the products of the M and S RNAs; both techniques are quite quick and easy, and provide excellent validation of results. In fact, we have already used the PAGE system to identify reassortants from non-mutagenized parents (see below).

(h) Classification of California serogroup viruses with monoclones

Although not a major goal of our work, it was of interest to look at the 3 subgroups of the California serogroup, according to their reactivity with our panel of monoclonal antibodies. As shown in the attached manuscript, the monoclonal panel (6 clones) suggested some inconsistencies with the conventional subgrouping of 11 viruses tested. The most striking exceptions were: (i) within the CE subgroup, LAC clones failed to react with CE virus; (ii) within the MEL subgroup both LAC and TAH clones reacted with JC virus; (iii) within the TVT subgroup, LAC clones reacted with TVT virus.

It is interesting to note that selected LAC and TAH clones are potentially useful reagents for identification of new field isolates. As shown in Appendix I, the use of 5 selected clones could potentially distinguish between the 7 different California serogroup viruses found in North America. In fact, Dr. Calisher, one of our collaborators with responsibility for the CDC arbovirus references laboratory, is seriously interested in using selected clones for this purpose.

(i) Variant viruses against individual monoclonal antibodies

With the panel of monoclonal antibodies we have generated, it is important to group them into different antigenic sites. Since the G1 protein is clearly of major biological and immunological importance, we have focussed our attention upon it. From work on other enveloped RNA viruses, particularly influenza and rabies virus, it may be predicted that the G1 protein carries a limited number of major antigenic sites (the order of 3-6).

One established strategy to group epitopes on a neutralizing protein is the selection of variant viruses. Variants may then be tested in both neutralization and in binding assays, to assign monoclonal antibodies to groups.

Dr. Gonzalez has utilized his panel of monoclonal antibodies to select a corresponding panel of variant viruses. TABLE 3 summarizes the protocol used to select variants, and the frequency with which they appeared, for some representative variants.

For most epitopes variants occurred at a frequency of about one per 10^5 to 10^6 pfu, which is similar to observed frequencies with influenza and rabies viruses.

A first series of cross-neutralization tests was then run to provide a preliminary look at patterns of neutralization.

TABLE 3. SELECTION OF ANTIGENIC VARIANTS OF LAC VIRUS
data of F. Gonzalez-Scarano, 1982

Monoclone Used to Select Variant		Frequency of Variants (Log_{10})	No. of Clones Prepared
Group	No.		
A (specific)	807-09	-3.7	1
	807-15	-6.2	1
	807-18	-5.3	1
	807-35	-5.3	3
	807-31	-6.0	1
	820-260	-5.4	1
B (cross-reactive)	807-12	-5.0	4
	807-22	-6.1	1
	807-33	-4.1	3
	813-13	-5.9	4
Median		-5.6	

* Ten-fold dilutions of LAC virus, strain original, tissue culture stock or clarified 10% brain homogenate, were incubated with a 1:10 dilution of ascites fluid (AF) for 30 minutes at 23C. They were then inoculated onto confluent BHK-21 cells in 6-well plates and overlayed with medium containing 0.5% agarose. Random plaques appearing at the end-point of the titration were picked, and purified a second time in the presence of the same monoclonal antibody. Frequency of variants was computed as $\frac{\text{titer of virus + AF}}{\text{titer of original stock.}}$

The results are summarized in Figure 2. It appears that variants begin to sort into two groups; those selected with LAC-specific monoclonal antibodies and those selected with cross-reactive antibodies. However, further work will be required to clarify and consolidate these emerging patterns.

To this end, Dr. Gonzalez has begun to derive "double variants", i.e., variants which have been selected by sequential growth in the presence of two different monoclonal antibodies. A current list of such variants is shown in TABLE 4.

(j) Next questions

The data reported above represent solid progress in the characterization of LaCrosse and Tahyna hybridomas. However, there are major gaps which require further work. These include

(i) Continuation of studies on grouping and mapping the G1 hybridomas, using variant viruses, neutralization tests, and binding assays.

(ii) Construction of G2 hybridomas.

(iii) The use of hybriodmas to localize the putative fusion function to G1 or G2.

(iv) Determination of the potential protective role of neutralizing and non-neutralizing G1 hybridomas.

Plans for such studies are set forth in the work proposed for the next year of this contract.

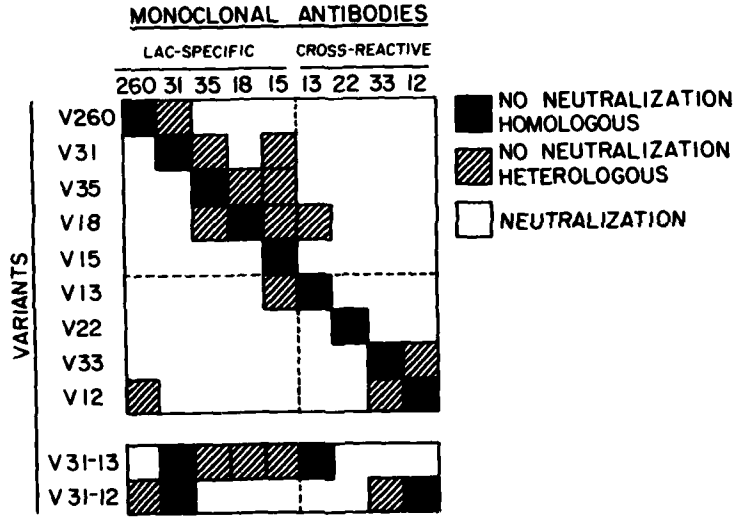


Figure 2.

Neutralization of variant variants by a panel of monoclonal antibodies. Each variant is designated by the monoclonal antibody (or antibodies) used to select it. Two fold dilutions of ascites fluid were combined with 100 pfu/0.1 ml of variant virus tissue culture stock, held at room temperature for 30 minutes, and inoculated (0.1 ml/well) on BHK-21 cells in 96-well plates. Presence of 100% CPE in 1:20 dilution of ascites fluid was taken as signifying no neutralization. Data of F. Gonzalez-Scarano.

TABLE 4. LIST OF DOUBLE VARIANTS SELECTED BY SEQUENTIAL USE OF TWO MONOCLONAL ANTIBODIES

data of F. Gonzalez-Scarano

First Monoclonal Antibody	Second Monoclonal Antibody	Number of Variants
807-31	813-13	1
807-31	807-09	1
807-31	807-12	1
807-33	807-35	1
820-260	807-35	1
807-22	807-35	1
807-12	807-35	1

3. Virulence of LaCrosse and Tahyna isolates

NOTE: As indicated above, we have designated the hybridoma studies as the specific object of this contract. However, the hybridomas will be used in experiments which are supported by our NIH grant AI 18085. Therefore, a brief account of the status of these experiments is set forth below.

The salient goal of our virulence and genetic studies is to correlate virus genes and their products (proteins) with biological properties of the virus, particularly virulence in rodents. The strategy which we are employing is to select and clone virus strains which demonstrate maximal differences in their virulence. The role of specific genes/gene products will then be analyzed in two ways: (a) construction of reassortant viruses using the selected clones as parents; (b) selection of hybridoma variant viruses, to look for the possible role of particular regions of the G1 molecule.

A further element in our plan is the selection of two viruses, LaCrosse (LAC) and Tahyna (TAH), as representative of more virulent (LAC) and less virulent (TAH) members of the California serogroup. Also of importance was the demonstration by Gentsch, Bishop and colleagues that LAC-TAH reassortants could be readily made.

(a) Standard LAC and TAH strains

To confirm that the standard strains of LAC (original) and TAH (Bardos 92) would exhibit characteristics similar to those published by Shope and others, we did age-specific titrations with the results shown in TABLE 5. These titrations were consistent with the published literature and confirmed that by the ip route, TAH virus is nonlethal in mice age 2 weeks or older. By contrast, LAC virus will kill older mice although it requires 10,000 - 100,000 suckling mouse LD50 to kill 50 - 100% of 4-week-old mice.

These results reinforce our original presumption that it would be preferable to have a LAC clone which showed greater ip virulence in weanling or adult mice or a TAH clone which showed less ip virulence in sucklings.

(b) Selection of an avirulent (non-neuroinvasive) strain

To obtain an avirulent TAH clone, we wrote Dr. Malkova in Praque, who shipped TAH strain 181/57. Malkova reported that this strain fails to kill suckling mice by the sc route.

Dr. Robert Janssen has tested the TAH/181-57 strain extensively, and found that it will serve very well as a prototype of an avirulent, i.e., non-neuroinvasive virus. As TABLE 5 indicates,

the TAH/181-57 virus possesses high intracerebral virulence in suckling and adult mice. Tested by a peripheral route, however, it differs dramatically from the virulent LAC/original strain. In suckling mice LAC/ori is highly virulent after intraperitoneal or subcutaneous injection whereas TAH/181-57 is very avirulent. Thus, by the subcutaneous route, LAC/ori requires 10 pfu per LD50 while TAH/181-57 requires 10^5 pfu per LD50.

TABLE 5. TRITRATIONS OF LAC AND TAH VIRUSES IN MICE

Assay	Log_{10} Titer per ml*		
	LAC/ori	TAH/B92	TAH/181-57**
pfu	7.2	ND	7.1
ic LD50			
1-3 days	7.1	6.3	7.3
3-4 weeks	6.5	6.2	ND
8-10 weeks	5.8	4.5	ND
ip LD50			
1-3 days	6.2	6.1	2.8***
1 week	ND	3.0	ND
2 weeks	ND	<1.0	ND
3-4 weeks	2.8	<1.0	ND
8-10 weeks	2.0	<1.0	ND

*LAC: LAC/original in BALB/c mice.
TAH: TAH/B92 and TAH/181-57 in outbred CD mice.
Undiluted: supernate of a 10% brain homogenate.
ND: not done

**Data of R. Janssen, 1982.

***subcutaneous injection.

TABLE 5A. TITERS AS PFU per LD50 in SUCKLING MICE

Assay route in Mice Age 1-3 Days	pfu per LD50			
	LAC/ori	TAH/B92	TAH/181-57*	LAC/ori V22**
ic	1	1	1	500
ip	3-10	3-10	ND	5,000
sc	10-100	ND	50,000	50,000

*Data of R. Janssen, 1982.

**Data of F. Gonzalez-Scarano, 1982.

(c) Pathogenesis of virulent LAC/ori and avirulent TAH/818-57

To compare virulent and avirulent viruses, sequential replication has been studied in suckling and adult mice. Preliminary in incomplete results are summarized in TABLES 6 and 7. These show that

(i) Both viruses replicate about equally well in the CNS although TAH/818-57 may multiply slightly more slowly than LAC/ori in the brains of adult mice.

(ii) LAC/ori replicates outside of the CNS in suckling mice, with high titers in blood and other tissues. By contrast, TAH/181-57 fails to replicate at all when injected sc, with little if any viremia or isolation from various peripheral tissues.

To localize the sites of virus replication and to better understand the difference between virulent and avirulent viruses, sequential immunofluorescent studies are being conducted by Dr. R. Janssen. Preliminary results show that

(iii) The extraneural replication site of virulent LAC/ori is restricted to straited and Cardiac muscle. There appears to be little if any replication in other mesodermal tissues such as chondroblastsor endothelium. Peripheral nerve fibers may be infected also, particularly the antonmic plexuses of the gut.

In summary, the key difference between virulent and avirulent viruses is the ability to replicate in striated muscle, and this appears to be the main source of viremia and account for neuroinvasiveness.

(d) Construction of reassortants from non-mutagenized parents

For genetic studies of virulence it was deemed advisable to avoid the use of reassortants constructed from ts mutants, since these reassortants have "silent" (non-ts) mutations in all their genes. Furthermore, experience with both bunyaviruses (laboratory of D.H.L. Bishop) and reoviruses (laboratory of B. Fields) has shown that such reassortants may show unpredictable aberrations in their biological properties due to such "silent" mutations.

We have begun to construct reassortants from the non-mutagenized prototype virulent (LAC/ori) and avirulent (TAH/181-57) viruses described above. BHK cells were infected simultaneously with cloned stocks of the two viruses (multiplicity of each at least 5 pfu per cell). Progeny have been cloned and working stocks grown from about 75 clones. These stocks were used to infect BHK cells and the viral polypeptides were pulse-labelled with S35 methionine. Infected cell lysates were analysed by PAGE and autoradiography. Of 75 clones, about 6 were tentatively identified reassortants with the phenotype

X-LAC-TAH

TABLE 6. SEQUENTIAL PATTERNS OF REPLICATION OF VIRUSES LAC/ori AND AVIRULENT TAH/181-57 FOLLOWING SC INJECTION OF SUCKLING CD MICE*
data of R. Janssen, 1983

Virus	Tissue	Log_{10} titer per mg tissue at days specified				
		1D	1.5D	2D	3D	3-4D (M)
LAC/ori	BRAIN	-	-	6	8	7
	SPLEEN	-	-	4.5	5	5
	LIVER	-	-	5	6	4.5
	HEART	-	-	5	4.5	4.5
	MUSCLE	-	-	4	5	4.5

		1D	2D	4D	6D	8D	10D
TAH/ 181-57	BRAIN	-	-	-	1.5	-	-
	SPLEEN	-	-	-	-	-	-
	LIVER	-	-	-	-	-	-
	HEART	-	-	-	-	-	-
	MUSCLE	-	-	-	-	-	-

*Titers determined on tissue pooled from 3 mice. M: moribund. -: less than 1.

TABLE 7. SEQUENTIAL REPLICATION OF VIRULENT LAC/ori and AVIRULENT TAH/181-57 in BRAINS OF ADULT CD MICE INOCULATED BY THE IC ROUTE*

data of R. Janssen, 1983

		Log_{10} titer per mg tissue at days specified					
Virus	Tissue	.75D	1D	2D	3D	4D	4-5D (M)
LAC/ori	BRAIN	1	4.5	5	5.5	6.5	6.5
	SPLEEN	ND	-	-	-	-	-
TAH 181-57	BRAIN	1.5	2.5	5	6.5	6.5	5.5
	SPLEEN	ND	-	-	-	-	1.5

*Titers determined on tissues pooled from 3 mice. ND: not done. M: moribund. -: less than 1.

This work is continuing and will be pressed forward in two directions: (a) the search for a simple method to determine the genotype of the L RNA (using high-resolution RNA gels or oligonucleotide fingerprinting); and (b) the attempt to obtain other reassortants such as X-TAH-LAC.

(e) Avirulence of selected variant viruses

Another incisive approach to identifying the molecular determinants of virulence is the use of variant viruses selected with monoclonal antibodies. If changes in certain epitopes alter virulence, then it will eventually be possible to specify virulence determinants at a sub-molecular level.

Using a standard ip challege of about 1000 pfu, F. Gonzalez has screened some of his variant viruses. Of these, preliminary data indicate that one variant (V22, see Figure 2, Table 5A) shows definite attenuation. Furthermore, it appears that this clone has reduced neurovirulence not just reduced neuroinvasiveness. This is an exciting result, since we can begin to explore the role of the G1 protein in determining two distinct virulence parameters.

Appendix I

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Characterization of Monoclonal Antibodies against the G1 and N Proteins of LaCrosse and Tahyna, Two California Serogroup Bunyaviruses

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Antibody-secreting hybrid cell lines were isolated by fusion of spleen cells from BALB/c mice infected with LaCrosse and Tahyna viruses with mouse myeloma cells. Of 23 cell lines, 15 secreted immunoglobulins which precipitated the G1 envelope glycoprotein, and 8 secreted immunoglobulins which precipitated the nucleocapsid protein; none reacted with the two other virion proteins, G2 or L. Monoclonal antibodies were characterized in neutralization (N), hemagglutination inhibition (HI), and ELISA tests against 11 California serogroup viruses. (i) Antibodies against the G1 viral glycoprotein fell into four groups designated A, B, C, and D. Groups A and B antibodies had high N and HI titers; group A antibodies were virus specific while those in group B were cross-reactive with many California serogroup viruses. Groups C and D antibodies had no N or HI activity, with one exception which did not neutralize but had HI activity. Group C antibodies bound preferentially to the homologous virus, while group D antibodies were cross-reactive. The concordance of N and HI responses suggests that the viral glycoprotein has a single domain which binds to cellular receptors and that groups A and B monoclonal antibodies interfere with this function. (ii) Nucleocapsid antibodies showed neither N nor HI activity and fell into two groups, designated E and F; group E antibodies bound preferentially to the homologous virus, while group F antibodies were cross-reactive with other California serogroup viruses. (iii) Monoclonal antibodies can be used to rapidly determine the phenotype of reassortants between LaCrosse and Tahyna viruses, for two of three gene segments. (iv) The identification of specific shared and unshared antigenic determinants provides a much improved rationale for the serologic taxonomy of California viruses, and suggests possible revisions in existing classification. Use of selected monoclonal antibodies also markedly facilitates identification of virus isolates.

INTRODUCTION

The California serogroup of bunyaviruses consists of 11 antigenically related viruses, often divided into three complexes, California encephalitis, Melao, and trivittatus, based mainly on neutralization (N)[2] and hemagglutination inhibition (HI) tests with polyclonal antisera (Sather and Hammon, 1967; Hubalik *et al.*, 1979; Bishop

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[2] Abbreviations used: BHK, baby hamster kidney cells, line 21; CDC, Centers for Disease Control, Vector-Borne Viral Diseases Division; CE, California encephalitis virus; DMEM-Hg, Dulbecco's modified Eagle's medium with 0.45 g glucose/100 ml; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; ELISA buffer, PBS with 2.04 g

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and Shope, 1980). The viral genome consists of single-stranded RNA divided into large (2.5×10^6 daltons), medium (1.8×10^6 daltons), and small (0.5×10^6 daltons) segments. In turn, these segments code for four structural proteins (Obijeski *et al.*, 1976; Gentsch *et al.*, 1977; El Said *et al.*, 1979; Bishop and Shope, 1980), the L RNA for an L protein (180×10^3 daltons, possibly an RNA polymerase); the M RNA for two glycoproteins (G1, 115–120 $\times 10^3$ daltons; G2, 38×10^3 daltons); and the S RNA segment for the nucleocapsid protein ($21–24 \times 10^3$ daltons).

Genetic reassortants are produced at high frequency in cultures coinfected with some pairs of California serogroup viruses (Gentsch and Bishop, 1976; Gentsch *et al.*, 1977; Gentsch *et al.*, 1980). In these studies the selection of reassortant viruses was enhanced by the use of ts mutant parents, and the genotype determined by the definitive but cumbersome method of RNA fingerprinting. We wished to construct reassortants using nonmutagenized parents and needed a method which would permit rapid determination of the genotype of large numbers of viral clones from doubly infected cultures. We therefore raised a panel of monoclonal antibodies against two prototype California serogroup viruses, LaCrosse (LAC) and Tahyna (TAH). As described below, selected monoclonal antibodies can be used to identify the phenotype of two of the three RNA genome segments.

The panel of monoclonal antibodies described herein represent, to our knowledge, the first set made against California serogroup viruses, and their application has begun to elucidate some of the functional and antigenic sites on two of the virion proteins. The present study identifies the G1 protein as the viral hemagglutinin; indicates that G1 is the major protein involved in virus neutralization; demonstrates virus-specific and shared antigenic determinants on both G1 and N proteins; and begins to put the serologic taxonomy of California viruses on a protein-specific and antigenic site-specific basis.

METHODS AND MATERIALS

Viruses. At UP and YARU, the following virus strains were used: LAC (original strain, passage 6); SSH (Burgdorfer P16); TAH (Bardos 92 P21); CE (BFS-283); SAN (20230 P7); INK (KN-3641 P5); JC (6IV-2235 P4); KEY (B64-55872 P8); MEL (TRVL P7); SDN (BeAr 103645 P3); TVT (Eklund P5). Suckling mouse brain pools (10%, w/v, in PBS with 0.75% bovine albumin, clarified by low-speed centrifugation, aliquoted, and stored at −70°) were utilized for all immunizations. Virus was grown in BHK cultures for preparation of ELISA antigen, as described below. At YARU, the same strains were used for HI tests.

At CDC, plaque reduction neutralization tests were conducted with the following virus strains: LAC (LaCrosse original P11); SSH (Burgdorfer P18); TAH (Bardos 92 P19); CE (BFS-283 P10); SAN (20230 P9); INK (KN-3641 P5); JC (6IV-2235 P10); KEY (B64-55872 P7); MEL (Tr 9375 P4); SDN (BeAr 103645 P4); TVT (Eklund P15).

Immunization of mice and preparation of spleen cells. BALB/c mice age 6 weeks were injected ip with 10^5 PFU of LaCrosse virus, a dose approximating one LD_{50}. After 4 weeks the surviving animals were

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NaCl/100 ml, 0.05% Tween 80, 1 m*M* EDTA, pH 7.2; GGFHS, gammaglobulin-free horse serum; GGFCS, gammaglobulin-free newborn calf serum; glycine SDS buffer, 50 m*M* Tris, 1% SDS, 380 m*M* glycine, pH 8.4; G1, virion glycoprotein; HAT, 0.1 m*M* hypoxathine, 0.01 m*M* aminopterin, and 0.03 m*M* thymidine in DMEM-Hg; HI, hemagglutination inhibition, ic, intracranial; Ig, immunoglobulin; INK, Inkoo virus; JC, Jamestown canyon virus; KEY, Keystone virus; LAC, LaCrosse virus; MEL, Melao virus; N protein, nucleocapsid protein; N antibody, neutralizing antibody; PBS, 0.85 g NaCl, 0.14 g Na_2HPO_4, 0.1 g NaH_2PO_4/100 ml, pH adjusted to 7.0; PEG, polyethylene glycol; PPO, diphenyloxazole; SA, San Angelo virus; SDN, Serro do Navio virus; SSH, snowshoe hare virus; TAH, Tahyna virus; TBS, 150 m*M* NaCl, 50 m*M* Tris, 0.5% NP-40, 0.1% bovine serum albumin, pH 8.0; TC7, African green monkey kidney cell line; TNE buffer, 0.01 *M* Tris, 0.1 *M* NaCl; 0.001 *M* EDTA; TVT, trivittatus virus; UP, University of Pennsylvania, Department of Microbiology; YARU, Yale Arbovirus Research Unit, Department of Epidemiology, Yale University.

boosted with 10^6 PFU ip and spleens were harvested 2-4 days later. For Tahyna virus immunization, 6-week-old BALB/c mice were given either 10^6 PFU ip or 10 PFU ic, and boosted 4 weeks later with 10^6 PFU iv, followed by sacrifice 2-4 days later. Spleens were removed aseptically, passed through a stainless steel mesh to make a cell suspension, treated briefly with 0.83% NH_4Cl to lyse erythrocytes, and washed in PBS.

Construction of hybridomas. Mouse myeloma cells, P3 × 63 Ag8 clone 653 (Kohler and Milstein, 1975; Koprowski *et al.*, 1977; Gerhard *et al.*, 1978; Kearney *et al.*, 1979) were maintained in DMEM-Hg with 15% (v/v) GGFCS, gentamycin 5 mg/100 ml, and 0.1 m*M* 8-azaguanine. One day prior to fusion, cell cultures were split 1:2 and azaguanine omitted. For fusion, a mixture of 10^8 spleen and 10^7 myeloma cells was made, pelleted by low-speed centrifugation, and the pellet resuspended in 1 ml PEG (40% polyethylene glycol 1000, Baker Chemical Co., in DMEM-Hg), held 5 min, pelleted, and resuspended in 60 ml HAT medium with 15% GGFCS, and distributed into 96-well Costar plates, 5×10^5 cells (0.1 ml) per well (Gerhard, 1980). In our experience, critical variables are the batch of PEG 1000, and the precise timing of the fusion protocol.

Following 2-3 weeks incubation at 37° in 6% CO_2, wells containing colonies were screened by ELISA assay. Positive colonies were cloned in 0.25% agarose (Seakem) in DMEM-Hg with 15% GGFCS, and individual colonies were grown in Costar T25 flasks in DMEM-Hg with 15% GGFCS, and supernates again tested by ELISA. Supernates were concentrated 10-fold by precipitation with 50% saturated ammonium sulfate at 4°, reconstitution, and extensive dialysis.

Ascitic fluids. BALB/c retired breeders were primed (Kennett *et al.*, 1980) by ip inoculation of 0.5 ml of Pristane (2,5,10,-14-tetramethyl pentadecane). Hybridoma cells (10^7 in 1 ml) were injected ip one month later, and ascitic fluid was harvested 1-2 weeks later, clarified by low-speed centrifugation, and stored at −20°.

ELISA assay. Antigens were prepared by growing viruses in BHK cells, harvesting supernates when severe cytopathic effect became evident, and clarifying at 10,000 *g* for 30 min. Virus was precipitated at 4° by adding 7 g PEG 6000 and 2.3 g NaCl/100 ml, stirring overnight, and centrifuging at 10,000 *g* for 30 min. The pellet was resuspended in 20 ml TNE buffer per 1000 ml original harvest, layered over 25% sucrose in TNE buffer, and centrifuged at 35,000 rpm in an SW-41 rotor for 2 hr. The final pellet was reconstituted in PBS with 50 m*M* $NaHCO_3$ (pH 9.5) to 1 ml per 100 original harvest, and this stock was stored at −70°.

Plastic 96-well plates (Cooke Micro ELISA) were seeded with 0.1 ml per well of stock antigen diluted in 50 m*M* $NaHCO_3$, pH 9.5, and held at 4° overnight. Titrations were used to determine the highest dilution which would give maximum antibody titer, usually 1:100 of the stock antigen. Thus, each well received the equivalent of $10^{7.5}$ PFU of original virus harvest. Plates were first rinsed five times with 0.005% Tween-80, followed by 10% GGF horse serum in ELISA buffer, held 30 min, washed five times, and drained. Test antibody was diluted in ELISA buffer (4% GGF horse serum was added to diluent when ascitic fluids were tested). After 2 hr at 37°, plates were washed five times with 0.005% Tween-80, and peroxidase-conjugated rabbit anti-mouse Ig (light and heavy chain-specific, Cappel Laboratory), diluted in ELISA buffer, was added. After one hour incubation at 37°, plates were washed with ELISA buffer, and substrate (0.03% tetramethylbenzidine in pH 4.5 citrate buffer with 0.012% H_2O_2) was added, 0.1 ml per well. A blue color developed in 15 min at 20° and plates were read by eye.

Immunoprecipitation. For labeling viral antigens, BHK cells were infected with LaCrosse and Tahyna virus at a multiplicity of about 1 PFU per cell, and incubated for 18 hr at 37°, when the medium was replaced with leucine-free or selective medium (Gibco), incubated for 45 min and [^{3}H]leucine or ^{3}H essential amino acids (Amersham) were added, using 200 μCi of amino acids (specific activity of 200 mCi/

mmol) per milliliter. After 4 additional hours incubation at 37°, cells were washed with cold PBS, and lysis buffer (150 *mM* NaCl, 5 m*M* EDTA, 2 *mM* phenylmethylsulfonylfluoride, 0.5% NP-40, 50 *mM* Tris-HCl, pH 7.4) was added, 10 ml per 75-cm^2 flask. The cell lysate was clarified by low-speed centrifugation and stored at −70°. Prior to immunoprecipitation the lysate was incubated for 30 min with staphylococci and clarified.

For monoclonal antibodies which bound staphylococcal protein A, cell lysate (10^5 cpm diluted to 0.1 ml) was incubated with 0.1 ml of monoclonal antibody concentrated from cell culture supernate or (less frequently) from ascitic fluid, for 90 min at 4°. Staphylococci (IgSorb, the Enzyme Center) were washed in TBS, pelleted, and the lysate-antibody mixture added to give a final 10% (v/v). The mixture was suspended and held 30 min at 23°, pelleted, washed twice in TBS with 2.5 *M* KCl, and 0.5% NP-40, and twice in TBS with 0.5% NP-40. The pellet was resuspended in 0.2 ml of Laemmli (1970) sample buffer, boiled 5 min, pelleted, and 0.025 ml of the supernate loaded on a gel.

For monoclonal antibodies which did not bind protein A, staphylococci were incubated with rabbit antiserum against mouse κ-light chains for 45 min, washed six times, and used as above.

Major problems in immunoprecipitation were poor labeling of the G2 viral protein, particularly with [^{3}H]leucine, and the nonspecific precipitation of several proteins, particularly when ascitic fluids were used, which was minimized by pretreatment of the cell lysate with the staphylococcal suspension.

Polyacrylamide electrophoresis. For electrophoresis, gels were composed of 10% acrylamide: 0.12% bis acrylamide, in a glycine-SDS buffer. Samples of 0.025 ml were loaded, electrophoresed at 30 mA until the marker was 10–12 cm into gel, fixed in 10% acetic acid in 45% methanol, dehydrated in DMSO, and infiltrated with 20% PPO in DMSO. Dried gels were exposed to Kodak XR-5 film at −70°, for 3-4 days.

Monoclonal isotype. The isotype of monoclonal antibodies was determined with the methods and reagents of W. Gerhard and D. Lopes, Wistar Institute. Polyvinyl chloride plates were loaded with 150 ng of viral antigen per well, dried overnight, washed in 10% GGFHS in PBS, and drained. Monoclonal antibody (0.025 ml of tissue culture supernate) was added, the plates were held 90 min at 23°, washed three times with PBS containing 0.08% sodium azide and 1% GGFHS. Isotype-specific rabbit anti-mouse Ig was added, the plates were held 90 min at 23°, and I^{125}-labeled goat anti-rabbit Ig was added (0.025 ml with 1000 cpm); the plates were held 90 min at 23° and washed four times in PBS. Wells were cut from the plate and counted in a gamma counter.

Neutralization tests. At UP, a quantal neutralization test was used, in which serial dilutions of antibody (0.1 ml) were added to 100 TCD50 of cell culture-propagated virus (0.1 ml). The mixture was held at 23° for 1 hr and inoculated into TC7 cell cultures in Costar microplates. Tests were read after incubation for 3 days at 37°.

At CDC, a plaque reduction method was used, as described elsewhere (Lindsey *et al.*, 1976).

Hemagglutination inhibition tests. The techniques of Clarke and Casals (1958) were used, with a micromodification. Ascitic fluids were extracted twice with acetone. Infected suckling mouse brain antigens were prepared by sucrose-acetone extraction and sonication (Ardoin and Clarke, 1976). Antigen-antibody mixtures were incubated 10 hr at 4°, then mixed with goose erythrocytes. The plates were incubated at 37° for 30 min prior to reading.

RESULTS

Isolation and Identification of Mouse Hybridoma Cell Lines Producing Monoclonal Antibodies against LaCrosse and Tahyna Viruses

BALB/c mice, age 6 weeks, were immunized by infection with LaCrosse or Tahyna viruses, as described under Meth-

TABLE 1

MONOCLONAL ANTIBODIES AGAINST THE G1 AND NUCLEOCAPSID PROTEINS OF LACROSSE (LAC) AND TAHYNA (TAH) VIRUSES. HOMOLOGOUS AND HETEROLOGOUS TITERS IN NEUTRALIZATION (N), HEMAGGLUTINATION INHIBITION (HI), AND ELISA TESTS

Protein	Immunizing virus	Group[a]	Hybridoma clone	Ig isotype	Homologous titers			Heterologous reactions		
					N	HI	ELISA	*N* (10 viruses)	HI (7 viruses)	ELISA (10 viruses)
G1	LAC	A	807-09	G2a	3.0[b]	2.9	>5.3	—[b]	—	ND[d]
			807-15	G2b	1.6	2.6	>5.3	—	—	ND
			807-18	G1	3.0	3.2	>5.3	—	—	ND
			807-31	G1	>2.5	3.2	>5.3	1[c]	1	ND
			807-35	G1	3.0	2.6	>4.7	—	—	ND
		B	807-12	G2a	>2.5	3.8	>5.3	5	6	ND
			807-22	G2a	1.9	3.2	>4.7	4	6	ND
			807-33	G2a	2.8	3.2	>4.7	5	6	ND
		C	807-21	G2a	—[b]	—	2.2	—	—	3
			807-25	G2b	—	—	4.6	—	1	—
			807-26	G2a	—	—	4.2	—	—	—
	TAH	B	813-13	G1	4.9	3.8	>4.7	7	6	ND
			813-48	G1	1.6	3.2	2.3	8	7	ND
			813-77	G1	4.9	4.4	>4.7	6	5	ND
		D	814-443	G1	—	3.2	4.5	—	7	1
Nucleocapsid	LAC	E	820-374	M	—	—	3.1	ND	ND	1
			807-28	G2a	—	—	3.5	ND	ND	2
		E/F	807-13	M	—	—	3.1	ND	ND	5
			807-32	M	—	—	3.7	ND	ND	4
	TAH	E	814-02	G2a	—	—	4.6	ND	ND	—
			814-08	ND	—	—	4.6	ND	ND	1
		F	814-47	M	—	—	2.2	ND	ND	10
			814-87	M	—	—	2.5	ND	ND	10

[a] Groups A through D, monoclonal antibodies against G1 protein. (A) Neutralizes homologous but not heterologous virus; (B) neutralizes homologous and heterologous viruses; (C and D) do not neutralize either virus; (C) heterologous ELISA titers against most California serogroup viruses are more than 0.3 log below homologous titer; (D) heterologous HI or ELISA titers for most California serogroup viruses are no more than 0.3 log below homologous titer; (E and F) monoclonal antibodies against nucleocapsid protein; (E) heterologous ELISA titers against most California serogroup viruses were more than 0.3 log below homologous titer; (F) heterologous titers against most California serogroup viruses were equal to or greater than homologous titer.

[b] All titers and indices shown as log —. Less than 1.3 (ELISA), less than 0.7 (N), less than 1.0 (HI), or negative against all heterologous viruses tested.

[c] The number of viruses against which the corresponding antibody reacted. The number tested is shown at the head of the column.

[d] Not done.

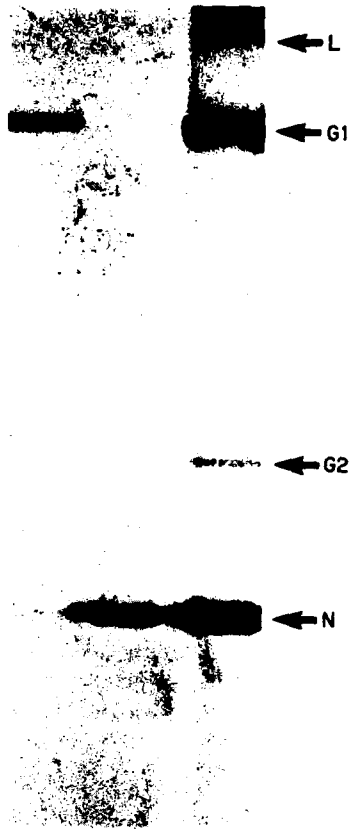

FIG. 1. Autoradiogram of a polyacrylamide gel in which labeled LaCrosse virus proteins were electrophoresed. Right lane: total infected cell lysate, showing the L, G1, G2, and nucleocapsid (N) proteins. Note that G2 usually labels weakly. Middle lane: lysate precipitated by monoclonal antibody 807-28, directed against the nucleocapsid protein. Left lane: lysate precipitated by monoclonal antibody 807-15 directed against the G1 protein.

ods and Materials. To minimize irrelevant immune responses, virus harvested from BALB/c mouse brain was employed for immunization.

Four successful fusions provided cell lines. Twenty-three cloned hybridomas were derived from over 400 initial microcolonies, of which about 100 were positive in the initial ELISA screen; after cloning in agarose, rescreening, passage as tumors, and screening of ascitic fluids, 23 cell lines remained.

The ELISA test, employing pelleted virions as antigen, was used throughout the screening process. Its specificity was demonstrated by negative tests with hybridomas directed against another virus (visna virus) and by the failure of bunyavirus hybridomas to react with control antigen prepared from mock-infected cultures (data not shown). No direct test was made of the sensitivity of the ELISA, but it clearly was able to detect antibodies directed to a surface (G1) and a core (N) protein. ELISA titers of ascitic fluids are shown in Table 1; these were usually 100- to 1000-fold higher than titers of corresponding cell culture fluids.

Precipitation of viral proteins and Ig isotypes. The protein specificity of each monoclonal antibody was demonstrated by immunoprecipitation of antigens derived from lysates of infected cell cultures labeled with ^{3}H-amino acids, exploiting the observation (Pennington *et al.*, 1977; Bishop and Shope, 1980) that synthesis is limited to viral proteins at 18 hr after infection with a multiplicity of 10 PFU per cell. Figure 1 shows that the label is concentrated in four viral proteins (right hand lane); the G2 viral protein is difficult to label, as others have observed (Bishop and Shope, 1980; Iroegbu and Pringle, 1981) and ^{3}H-amino acids or [^{35}S]methionine gave better results that [^{3}H]leucine. To obtain monoprotein precipitation it was necessary to use cell culture fluids, rather than ascitic fluids, and to pretreat the infected cell lysate with staphylococci. Under these conditions each monoclonal antibody cleanly precipitated a single viral protein, as shown in Fig. 1 (G1, left lane; nucleocapsid, middle lane).

Monoclonal antibodies were isotyped by a radioimmunoassay which clearly classified each antibody (Table 1). Monoclonal antibodies against the G1 viral glycoprotein were IgG1 (7), IgG2a (6), and IgG2b (2), which is generally representative of isotype distribution in mouse serum. By contrast, monoclones against the nucleocapsid protein (five were IgM and two were IgG2a) were atypical for a secondary immune response.

Reactivity Pattern of Anti-G1 Monoclonal Antibodies in Neutralization (N), Hemagglutination Inhibition (HI), and ELISA Tests

Monoclonal antibodies against the G1 viral glycoprotein could be classified into four groups, designated for convenience A, B, C, and D (Table 1). Anti-G1 antibodies classified as A or B had high titers in N and HI test, as well as in the ELISA assay. Group A monoclones had considerable N and HI titers against the homologous virus but showed little or no reactivity with other California serogroup viruses. An exception was antibody 807-31, which reacted only with homologous LAC and SSH, a virus known to be closely related antigenically to LAC virus (Bishop and Shope, 1980). Group B monoclonal antibodies, in both N and HI tests, reacted with the homologous virus and with many (but not all) California serogroup viruses.

Group C and D monoclonal antibodies reacted in the ELISA assay but did not neutralize (Table 1). There were only four such antibodies, of which three were classified as C and one as D. Group C antibodies were type specific in that their ELISA titers were considerably higher (usually at least 10-fold) against homologous virus, but they did show low titers against many heterologous viruses (data not shown). The group D antibody had a high HI titer against all heterologous California serogroup viruses tested.

There was a striking correlation between N and HI reactivity of monoclonal antibodies against the viral G1 protein (Table 1). Thus, 11 antibodies had high N and HI titers, while 3 antibodies were negative in both tests. Clone 814-443 constituted the sole exception; this antibody showed a high HI titer against all California serogroup viruses tested, but failed to neutralize any virus.

Reactivity pattern of anti-nucleocapsid monoclonal antibodies in ELISA tests. Monoclonal antibodies against the nucleocapsid protein, as expected (Gentsch *et al.*, 1980), failed to neutralize and, as predicted (Shope, personal communication, 1981) failed to inhibit hemagglutination. In the ELISA assay (Table 1), anti-nucleocapsid antibodies were classified as group E (homologous titer more than twofold greater than heterologous titer), group F (many heterologous titers equal to homologous titer), or E/F (intermediate pattern). Of eight anti-nucleocapsid antibodies, four were group E (reacted with 0, 1, or 2 of 10 heterologous viruses), two were group F (reacted with all 10 heterologous viruses), and two were group E/F (reacted with 4 or 5 viruses).

Use of monoclonal antibodies for rapid phenotyping of reassortant viruses. From the foregoing data, it appeared that a few selected monoclones could be used for phenotyping of reassortant viruses. Prototype tests were conducted with parental and reassortant viruses. Table 2 shows that two virus-specific anti-G1 monoclonal antibodies differentiated viruses bearing the LAC M RNA segment from viruses bearing the TAH M RNA segment, in N and HI tests. The same distinction can be made in ELISA titrations, although the present panel lacks a highly type-specific TAH monoclonal antibody.

Table 3 shows that, in the ELISA test, two virus-specific anti-nucleocapsid monoclonal antibodies clearly distinguish viruses bearing the LAC S RNA segment from viruses bearing the TAH S RNA segment. Figure 2 illustrates such a test.

DISCUSSION

Rapid Phenotyping of Reassortant Viruses with Monoclonal Antibodies

The availability of type-specific monoclonal antibodies should make it feasible to screen viral clones from doubly infected cell cultures and rapidly determine a tentative phenotype for M and S RNA segments. The simplest approach will probably utilize the ELISA assay, although microneutralization could be used to confirm the phenotype of the G1 protein. Selected reassortant viral clones could then be typed for the L RNA segment by oligonucleotide fingerprinting.

TABLE 2

DETERMINATION OF THE PHENOTYPE OF THE G1 PROTEIN (M RNA SEGMENT) OF REASSORTANT LACROSSE (LLL) AND TAHYNA (TTT) VIRUSES BY SELECTED MONOCLONAL ANTIBODIES AGAINST THE G1 VIRAL PROTEIN

Virus genotype	ELISA titer		N titer		HI titer		Putative G1 phenotype
	807-35[b]	813-77	807-31	813-77	807-09	813-77	
LLL	>4.6[c]	>4.6	>4.0	1.6	2.9	2.6	
TLT[a]	>4.6	>4.6	>4.0	1.9	2.9	>2.9	L
LTL[a]	1.9	>4.6	<0.7	4.0	<2.0	4.1	T
TTT	<1.3	>4.6	<0.7	4.0	<1.0	4.4	

[a] Reassortant viruses, provided by D. H. L. Bishop, were genotyped by RNA fingerprints (Gentsch *et al.*, 1980).

[b] Identification number of hybridoma clone (Table 1).

[c] All titers shown as log.

Distribution of Monoclonal Antibodies by Protein Specificity and by Isotype

The distribution of monoclonal antibodies by protein specificity and by isotype showed two striking features, the absence of antibodies against the G2 viral protein and the high frequency of IgM isotypes among the anti-nucleocapsid antibodies.

Since G2 is a surface glycoprotein, and since it is represented equally with G1 (Bishop and Shope, 1980) at about 600 molecules per virion, it would be expected that anti-G2 would be well represented in the immune response repertoire of infected mice and therefore among a panel of hybridomas.

The unexpected under-representation of certain proteins in a panel of anti-viral monoclonal antibodies has been reported with other viruses. For instance, working with influenza virus, Gerhard and colleagues (1980) found very few anti-neuraminidase antibodies, only one for every 25 anti-hemagglutinin antibodies. Likewise, Nowinski and co-workers (Lostrom *et al.*, 1979) noted a paucity of anti-gp 70 monoclonal antibodies following immunization with murine leukemia viruses and resorted to virus-infected cells as an immunogen to raise the desired antibodies.

There are several possible explanations for the absence of G2 antibodies. It seems almost certain that the G2 protein is perserved in preparing the concentrated virus used as antigen, since the G2 is readily visualized in infected cell lysates (Fig. 1)

TABLE 3

DETERMINATION OF THE PHENOTYPE OF THE NUCLEOCAPSID PROTEIN (S RNA SEGMENT) OF REASSORTANT LACROSSE (LLL) AND TAHYNA (TTT) VIRUSES BY SELECTED MONOCLONAL ANTIBODIES[a]

Virus genotype	ELISA titer		CF titer[b]		Putative nucleocapsid phenotype
	807-28	814-02	807-28	814-02	
LLL	>4.6[c]	1.6	3.1	<2.2	
LTL[a]	>4.6	1.9	2.8	<2.2	L
TLT[a]	2.2	>4.6	2.2	2.8	T
TTT	2.2	>4.6	2.2	2.8	

[a] See footnote to Table 2.

[b] Complement fixation.

[c] All titers shown as log.

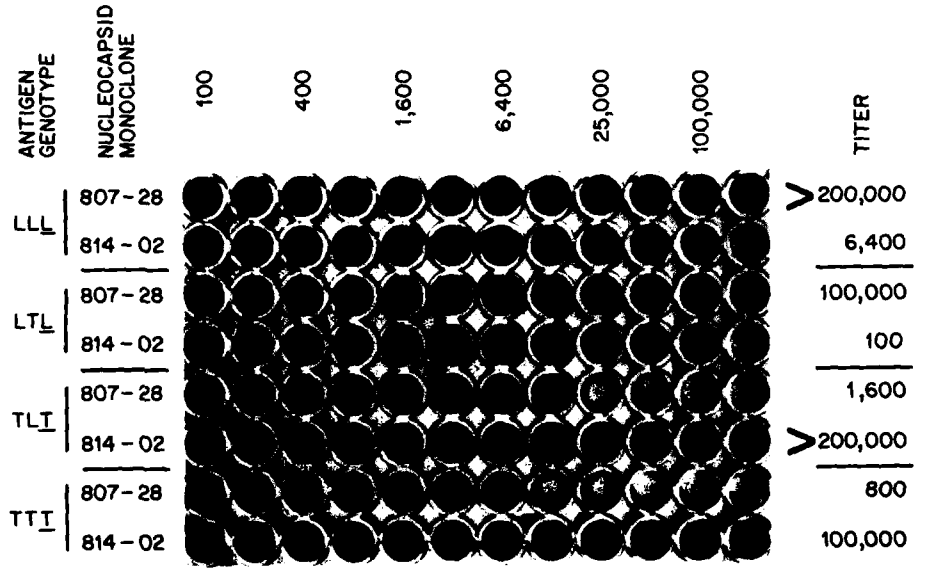

FIG. 2. Identification of the nucleocapsid protein (S RNA segment) of parent LAC (LLL) and TAH (TTT) viruses and of reassortants, in the ELISA assay. Antigen was prepared for each of the four viruses and coated onto two rows of wells. Two monoclonal antibodies, 807-28 and 814-02, were titrated, each in single rows from 100 to 200,000. The titers are shown at right and indicate that viruses with a LAC S RNA had a "high-low" titer pattern while viruses with a TAH S RNA had "low-high" titer pattern. Reassortant viruses were provided by D. H. L. Bishop.

and in centrifuge-concentrated virions (Kingsford and Hill, 1981a, b) similar to those used as ELISA antigen. Perhaps the ELISA antigen may bind G2 antibodies poorly. It has been shown (Lee *et al.*, 1981a) that binding assays may fail to register certain antibodies which, nevertheless, can precipitate reoviral proteins. Alternatively, the immunization protocol, utilizing active infection, may favor stimulation of an anti-G1 response with little anti-G2 response in the absence of hyperimmunization. In any event, the use of purified G2 protein as immunogen or as ELISA antigen, combined with the testing of individual mice prior to fusion, should yield anti-G2 hybridomas.

The high frequency of the IgM isotype among nucleocapsid hybridomas could reflect a disposition of identical antigenic determinants in close array, as occurs with polysaccharide antigens. Such an antigen binds IgM pentamers at multiple sites, and this would favor the detection of IgM antibodies in the ELISA screen (J. Rodwell, personal communication, 1981). *In vivo*, the same antigen presentation could preferentially stimulate an IgM response.

Functional Sites on the Viral G1 Glycoprotein

From the data reported herein, we can begin to delineate functional sites on the viral G1 glycoprotein. A tentative interpretation and synthesis appears in Fig. 3.

(i) The G1 molecule clearly acts as the viral hemagglutinin. Furthermore, one domain on the molecule carries this function and antibody directed to distant sites on G1 does not inhibit hemagglutination.

(ii) The close correlation of neutralizing and HI activity of monoclonal antibodies strongly suggests that the domain which binds to erythrocytes also binds to receptors on virus-susceptible cells in culture. The one hybridoma (S14-443) with high HI

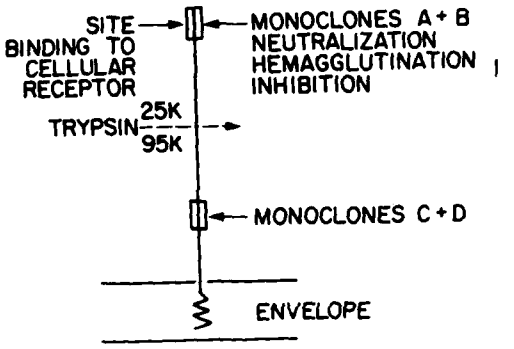

FIG. 3. A tentative diagrammatic scheme to show functional sites on the G1 glycoprotein of California serogroup viruses. Based on the present data and on the work of Kingsford and Hill (1981a).

activity and no neutralizing activity suggests that binding sites, although overlapping, may differ somewhat in structure or function.

Reovirus offers an interesting contrast. A single outer capsid protein, sigma 1, is responsible for attachment to virions to cell receptors (Weiner *et al.*, 1978; Lee *et al.*, 1981a, b). However, the domains involved are somewhat separate since anti-sigma 1 antibodies possess either neutralizing or HI activity, but rarely both (Burstin *et al.*, 1981).

(iii) There is a second antigenic site on the G1 viral glycoprotein which is sufficiently distant from the putative receptor-binding site so that antibodies against this site will not block attachment to cells.

These observations are congruent with the data of Kingsford and Hill (1981a, b), who have studied the effect of proteolytic enzymes upon virion function. Certain enzymes cleave a polypeptide of 20,000-50,000 daltons from the G1, making the virus nonneutralizable. The major G1 moiety, left after proteolysis, can still bind antiviral antibody. In light of these observations, it seems likely that the receptor-binding site is situated on the outer 20,000-50,000 segment of the G1 segment of the G1 and that our nonneutralizing hybridomas bind to the enzyme-resistant base of the molecule.

The function of the G2 molecule remains obscure. Almost certainly it is not involved in attachment to erythrocytes or to cellular receptors, since Kingsford and Hill (1981a) have found that polyclonal anti-G2 antiserum will not neutralize and since pronase or bromelain, which leave G2 intact while degrading G1 markedly, renders the virus totally noninfectious. The ability of selected anti-G1 monoclonal antibodies to prevent hemagglutination and to render the virus noninfectious also implies that the G2 protein is not involved in attachment to receptors.

The major function yet to be assigned to a glycoprotein is fusion. Extrapolating from the paramyxoviruses, where receptor-binding and fusion are carried by different glycoproteins (Choppin *et al.*, 1981), we would expect that if this function was mediated by G2, anti-G2 antibody would neutralize. Further elucidation of the role of G2 awaits the availability of anti-G2 monoclonal antibodies.

Biological Functions of Antiviral Antibody

The present panel of hybridomas makes it possible to investigate the role of different antibodies in the host response to infection. Of particular interest is the effect of nonneutralizing anti-G1 antibodies. For instance, if any of these (814-443 would be a candidate) block neutralization, what effect would they have when administered prior to or during infection? Studies of this kind may provide new insights into the molecular basis of diverse effects of anti-viral antibody.

Use of Monoclonal Antibodies for Antigenic Taxonomy of California Serogroup Viruses

Availability of monoclonal antibodies makes it possible to examine the antigenic taxonomy of California serogroup viruses on a protein-specific and on a test-specific basis. Antibody titers against 11 California serogroup viruses (data not shown) suggest several generalizations. The nucleocapsid antibodies demonstrate common antigenic sites on the nucleocapsid protein, but do not appear to make fine

TABLE 4

A SCHEME FOR THE DIFFERENTIATION OF NORTH AMERICAN MEMBERS OF CALIFORNIA SEROGROUP BUNYAVIRUSES USING MONOCLONAL ANTIBODIES IN THE NEUTRALIZATION TEST

Hybridoma clone	Virus						
	LAC[a]	SSH	JC	TVT	SAN	CE	KEY
807-09	+[b]	−	−	−	−	−	−
807-31	+	+	−	−	−	−	−
807-33	+	+	+	+	−	−	−
807-12	+	+	+	+	+	−	−
807-13	+	+	+	−	+	+	−

[a] LAC, LaCrosse; SSH, showshoe hare; JC, Jamestown Canyon; TVT, trivittatus; SAN, San Angelo; CE, California encephalitis; KEY, Keystone.

[b] +, Monoclonal antibody neutralizes the corresponding virus.

distinctions between various California viruses.

The anti-G1 monoclonal antibodies appear useful for defining both relationships and differences among California serogroup viruses. Of the three tests used, the ELISA and HI tests effectively demonstrate antigenic determinants shared by all members, while neutralization appears to be the most useful for separating and grouping individual members within the California serogroup.

The six cross-reactive neutralizing monoclonal antibodies raised against LaCrosse or Tahyna viruses are shown in Table 1. When tested against 11 California serogroup viruses (data not shown) several points emerge. These antibodies neutralize the six viruses of the CE complex (LAC, SSH, TAH, CE, SAN, INK) except that LaCrosse antibodies fail to neutralize CE virus. The remaining viruses tested belong to the Melao complex (JC, MEL, KEY, SDN) or the trivittatus complex (TVT). Surprisingly, LaCrosse and Tahyna monoclonal antibodies neutralize JC and TVT viruses as frequently as they neutralize viruses within the CE complex.

Use of Monoclonal Antibodies for the Identification of the California Serogroup Viruses of North America

To determine the potential usefulness of the present panel of monoclones in identification of new field isolates, a subset of data (Table 4) was extracted from the detailed results (not shown). Neutralization tests, as noted, show the highest degree of specificity and five neutralizing antibodies were selected from the panel. These were tested against the seven California serogroup viruses occurring in North America. Inspection of Table 4 indicates that each of the seven viruses exhibits a different pattern.

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Appendix 2

MONOCLONAL ANTIBODIES AGAINST THE G1 AND NUCLEOCAPSID PROTEINS OF LACROSSE AND TAHYNA VIRUSES

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ABSTRACT

Monoclonal antibodies against the Gl (glycoprotein) and Nc (nucleocapsid) proteins of LaCrosse (LAC) and Tahyna (TAH) viruses were generated using a standard protocol. Monoclonal antibodies against Gl were either neutralizing or non-neutralizing, and there was close concordance between inhibition of hemagglutination and neutralizing activities. The LAC virus neutralizing antibodies could be further subdivided into strain-specific and cross-reactive groups on the basis of both neutralization and inhibition of hemagglutination. These data support the concept of a glycoprotein molecule with three antigenic sites, two involved in neutralization and hemagglutination, and an additional site with no biological function as yet defined. Preliminary information from LAC virus variants selected with these monoclonal antibodies agrees with this interpretation. Anti Nc antibodies were all cross-reactive in an ELISA assay. Patterns of monoclonal cross-reactivity against California serogroup viruses are compared with the currently accepted antigenic relationships.

INTRODUCTION

Monoclonal antibodies have been instrumental in the delineation of the functional and antigenic sites of the glycoproteins of both RNA (Gerhard et al. 1981), and DNA (Pereira et al. 1980) viruses. This has been accomplished by the use of antibodies to identify specific biological functions, by the selection of monoclonal virus variants, and by binding analysis. Bunyaviruses, with two glycoproteins and an otherwise simple structure, are amenable to study by these methods.

Monoclonal antibodies to two prototype California serogroup viruses, LaCrosse (LAC) and Tahyna (TAH), were generated by adaptation of standard methods (Gonzalez-Scarano et al. 1982). Mice were immunized with an active infection, re-challenged, and their splenic cells fused with non-secreting, HAT-sensitive myeloma cells (Kohler and Milstein 1975, Koprowski et al. 1977; Kearney et al. 1979). Colonies growing in HAT medium were selected for antibody secretion with an enzyme-linked immunosorbent assay (ELISA) using partially purified virus as antigen, and were then cloned in semi-soft agarose (Kennett et al. 1980). Four fusions yielded 24 lines which secreted antibodies directed against either the major glycoprotein (Gl) or the nucleocapsid (Nc) protein (Figure 1, Table 1).

Fig. 1. Confluent BHK-21 cells were infected with approximately one pfu per cell of either LAC or TAH virus. Fifteen hours later the medium was replaced with leucine-free medium and ^{3}H leucine added for 2-4 hours. The cell extracts were immunoprecipitated with monoclonal antibody and Staphylococci (Group A, Cowan I strain) with and without rabbit anti-mouse immunoglobulin. Alternatively, virus was grown in the presence of ^{35}S methionine and purified by polyethylene glycol 6000 precipitation and sucrose gradient centrifugation, and then used for immunoprecipitation after lysis with 0.5% NP-40. The samples were boiled in sample buffer and run on a 10% Polycrylamide gel (Laemmli 1970). A. Precipitation of Gl protein by two anti-Gl monoclonal antibodies. The first (left) lane shows purified LAC virus. B: Precipitation of Nc protein by monoclonal antibody. The first (left) lane shows LAC-infected cell extract.

Table I

Protein Specificity and Isotype of 24 Monoclonal Antibodies Generated against LaCrosse (LAC) and Tahyna (TAH) Viruses**

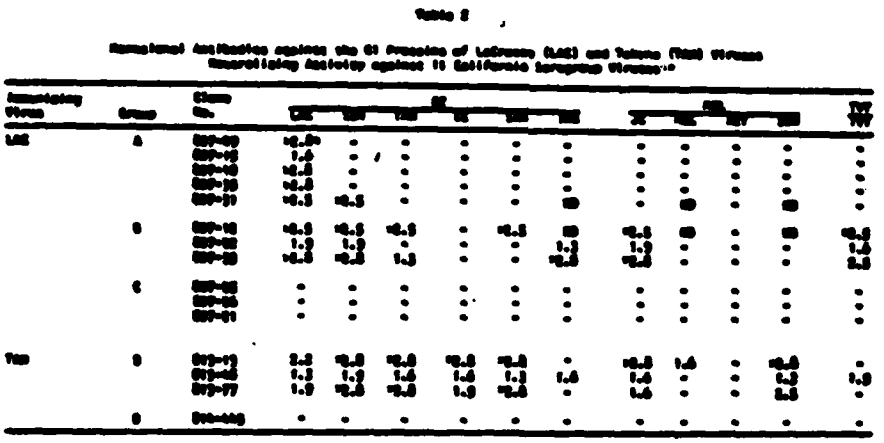
Parameter	Protein specificity of monoclone*			
	G1	G2	Nc	L
Immunizing Virus				
LAC	12	-	4	-
TAH	04	-	4	-
Isotype				
IgG1	07	-	-	-
IgG2	09	-	2	-
IgM	—	-	6	-
Totals	16	-	8	-

*Protein: G1, G2: glycoproteins. Nc: nucleocapsid. L: large protein.

**6-week old Balb/c mice were injected with an intracranial (TAH) or intraperitoneal (LAC) dose equivalent to one LD50. The surviving animals were boosted with 10 LD50 either iv (TAH) or ip (LAC) and their spleens harvested 2-4 days later. 10^8 spleen and 10^7 myeloma cells were fused with 40% polyethylene glycol 1000 and hybrid cells selected in HAT medium. Screening was done with an ELISA assay and positive colonies were cloned in semi-soft agarose. Isotyping of monoclonal antibodies was done by means of a radioimmunoassy using type-specific rabbit anti-mouse immunoglobulin and I^{125} labelled goat anti-rabbit Ig.

ANTI-G1 (GLYCOPROTEIN) ANTIBODIES

Twelve of 16 antibodies directed against the G1 glycoprotein of either TAH or LAC virus were capable of neutralization in plaque reduction and microneutralization assays. The neutralizing antibodies were tested against 11 California serogroup viruses (one antibody not tested) and divided into strain specific or cross-reacting groups on the basis of these results (Table 2). Specific antibodies were only obtained from the mice that had been immunized with LAC virus, and ascites fluids made with these had high neutralizing titer against LAC virus yet showed no neutralization of other California viruses. All of the antibodies obtained by immunization of mice with TAH virus, and some of those generated by LAC virus neutralized several California serogroup viruses.



Ascites fluids carrying these anti-G1 monoclonal antibodies were then tested for hemagglutination inhibition activity using 8 California serogroup antigens. The results (Table 3) show correlation between neutralization and hemagglutination inhibition for 14 of 15 monoclonal antibodies. The one exception is antibody 814-443, which, while inhibiting hemagglutination using most of the antigens tested, was unable to neutralize any of these viruses.

Table 3

Monoclonal Antibodies against the G1 Proteins of LaCrosse (LAC) and Tahyna (TAH) Viruses
Hemagglutination Inhibition (HI) Activity against 8 California Serogroup Viruses[aa]

Immunizing Virus	Group	Clone No.	CF					HI		PRN
			LAC	SSH	TAH	CE	SSE	JC	KEY	TVT
LAC	A	827-09	2.3[a]	-	-	-	-	-	-	-
		827-15	2.6	-	-	-	-	-	-	-
		827-16	3.2	-	-	-	-	-	-	-
		827-25	2.6	-	-	-	-	-	-	-
		827-31	3.2	3.2	-	-	-	-	-	-
	B	827-12	3.8	4.4	3.8	3.2	2.6	4.4	-	2.6
		827-22	3.2	3.8	3.5	3.8	3.2	3.8	3.2	-
		827-33	3.2	3.8	3.8	2.6	2.3	3.8	-	2.6
	C	827-05	-	2.0	-	-	-	-	-	-
		827-26	-	-	-	-	-	-	-	-
		827-21	-	-	-	-	-	-	-	-
TAH	B	813-13	2.3	3.8	3.8	3.8	3.2	3.6	-	-
		813-48	3.2	3.2	3.2	3.2	3.2	3.2	2.6	2.6
		813-77	2.6	2.6	4.4	2.6	2.6	2.8	-	-
	B	814-443	3.2	3.8	3.2	3.2	3.2	3.2	-	2.6

[a] Titers as $\log_{10}$. -: less than 1.2.

[aa] Infected suckling mouse brain antigens were prepared by sucrose-acetone extraction and sonication. Ascites fluids were extracted twice with acetone, diluted and mixed with antigen for 18 hours at 4C. Goose erythrocytes were added, and the plates were incubated at 37C for 30 minutes prior to reading (Clarke and Casals, 1958; Arnaiz and Clarke, 1967).

Group C and D antibodies immunoprecipitated G1 but did not neutralize; these were also unable to inhibit hemagglutination, except for 814-443. Group C and D antibodies were tested by ELISA with antigens prepared from 11 viruses (Table 4). All of these antibodies bound antigens from most California serogroup viruses, but heterologous titers were usually considerably lower than the homologous titer.

Table 4

Neutralizing Monoclonal Antibodies against the E2 Proteins of LaCrosse (LAC) and Tahyna (TAH) Viruses
ELISA Tests with 11 California Serogroup Viruses*

Immunizing Virus	Clone No.	Group	E2						NS1				TVT TVT
			LAC	JAM	TAH	CE	SSH	TRI	JC	REL	KEY	INK	
LAC	807-25	C	4.6**	2.5	2.2	1.6	2.2	1.3	1.9	<1.3	<1.3	1.3	2.8
	807-26		4.2	3.1	2.5	2.5	3.6	1.6	3.7	1.6	2.2	1.6	3.1
	807-31		2.2	1.6	1.3	2.8	1.9	1.6	1.6	<1.3	<1.3	1.3	2.5
TAH	816-443	B	4.3	2.2	4.5	<2.1	2.5	2.5	3.7	2.2	4.0	2.2	3.1

* Titers shown as $\log_{10}$.

** Antigens were prepared by concentrating virus with PEG 6000 and pelleting through a cushion of 25% sucrose in TNE buffer followed by resuspension of the pellet in PBS with 50mM $NaHCO_3$ (pH 9.5). ELISA plates were inoculated with antigen at 4C overnight and washed, blocking remaining protein-binding sites with human serum. Ascites fluid dilutions were added, incubated at 37C for 2 hours, followed by addition of peroxidase-conjugated rabbit anti-mouse Ig. After 1 hour further incubation at 37C, substrate (0.03% tetramethylbenzidine, in pH 4.5 citrate buffer with 0.012% H_2O_2) was added, and plates read visually (Gonzalez-Scarano et al. 1982).

LAC ANTIGENIC VARIANTS

Antigenic variants of LAC virus were selected with the neutralizing monoclonal antibodies. The variants were present (Table 5) at frequencies ranging from $10^{-3.7}$ to $10^{-6.2}$, with a median frequency of $10^{-5.6}$, a figure which is consistent with the frequency of antigenic variants of other RNA viruses (Holland et al, 1982). Most of the variants were obtained from a mouse brain homogenate pool that served as the standard inoculum. Antibody 807-31 completely neutralized this brain stock, but we were able to select variants with this antibody from a cell culture stock of the same strain. Variants were tested for neutralization by the entire panel of monoclonal antibodies. Each variant was, by definition, not neutralized by the monoclonal antibody used to select it. In addition, some variants escaped neutralization by other monoclonal antibodies in the panel (Figure 2).

Table 5

Selection of Antigenic Variants of LAC Virus

Monoclone Used to Select Variant		Frequency of Variants
Group	No.	(Log10)
A (specific)	807-09	-3.7
	807-15	-6.2
	807-18	-5.3
	807-35	-5.3
	807-31	-6.0
	820-260	-5.4
B (cross-reactive)	807-12	-5.0
	807-22	-6.1
	807-33	-4.1
	813-13	-5.9
Median		-5.6

*Ten-fold dilutions of LAC virus, strain original, tissue culture stock or clarified 10% brain homogenate, were incubated with a 1:10 dilution of ascites fluid (AF) for 30 minutes at 23C. They were then inoculated onto confluent BHK-21 cells in 6-well plates and overlayed with medium containing 0.5% agarose. Random plaques appearing at the end-point of the titration were picked, and purified a second time in the presence of the same monoclonal antibody. Frequency of variants was computed as $\frac{\text{titer of virus + AF}}{\text{titer of original stock.}}$

While these data are preliminary, Figure 2 demonstrates two points: (1) all the initial variants are unique in that they show different patterns when tested against this group of neutralizing antibodies, and (2) variants selected with group A antibodies escape neutralization only by other group A monoclones, while Group B variants escape neutralization by group B antibodies. Antibody 813-13 and its variant are an exception.

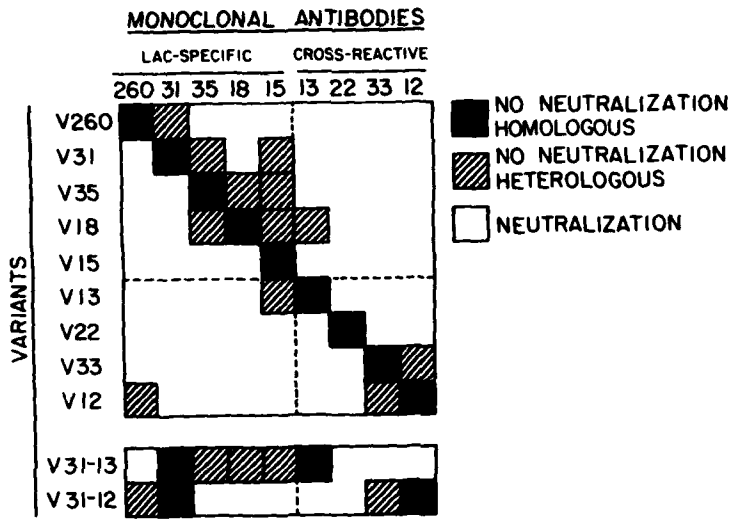


Fig. 2. Neutralization of variant variants by a panel of monoclonal antibodies. Each variant is designated by the monoclonal antibody (or antibodies) used to select it. Two fold dilutions of ascites fluid were combined with 100 pfu/0.1 of variant virus tissue culture stock, held at room temperature for 30 minutes, and inoculated (0.1 ml/well) on BHK-21 cells in 96-well plates . Presence of 100% CPE in 1:20 dilution of ascites fluid was taken as signifying no neutralization.

We interpret these results as suggesting that G1 has at least three separate antigenic sites. Two sites are close to the receptor part of the molecule and binding to them neutralizes and inhibits hemagglutination by the virus. One of these sites is strain specific, at least in LAC virus, and the other site is common to all members of the California serogroup. The variant virus data, preliminary as it is, supports this concept of two, relatively non-overlapping sites. A third site on G1 is further removed from the receptor site, and binding to it does not neutralize the virus. Confirmation of the independent identity of this site will be achieved if we can demonstrate that antibodies to this site bind to variants as well as to the parent virus. Kingsford (1983) has found that trypsin cleavage of G1 destroys the neutralizing site yet leaves the non-neutralizing site intact.

Our failure to obtain anti-G2 monoclonal antibodies has now been experienced by two other workers (Kingsford, 1983; Grady, personal communication 1982). Possible explanations include: (1) Our screening method may not detect anti-G2 antibodies, even though our ELISA antigen is prepared with whole virions. (2) There is a preferential immune response to the G1 glycoprotein. There is precedent, in the influenza system, for a skewed response against one of two glycoprotein spikes (Gerhard et al. 1980). Preliminary work by J. Gentsch (Gentsch, personal communication 1982) using a G2 enriched preparation as an ELISA antigen, suggests that the immune response to G2 is not as prominent as the response to G1 and Nc.

NUCLEOCAPSID ANTIBODIES

A number of monoclonal antibodies binding the Nc protein were produced with both LAC and TAH viruses (Table 6). They were predominantly of the IgM isotype. These antibodies did not neutralize the virus or inhibit hemagglutination. ELISA tests showed that some antibodies bind preferentially to the homologous antigen, while others bind to many California serogroup viruses.

Table 6

ANTIGENIC COMPARISON of CALIFORNIA SEROGROUP VIRUSES

Neutralizing monoclonal antibodies reported here support the conventional classification among the California serogoup viruses into three subgroups; i.e. three complexes, California encephalitis, Melao, and Trivitattus. The main exception is Jamestown Canyon virus, which by these assays appears to be more closely related to the California encephalitis complex than to the Melao complex. The anti-Nc antibodies identify shared antigenic determinants and are not particularly useful for fine discrimination within the California serogroup.

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